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1. Your reference	RJE/RSG/PG4744
2. Patent application number *(The Patent Office will fill in his part)*	**0201735.8** 25 JAN 2002
3. Full name, address and postcode of the or of each applicant *(underline all surnames)*	Glaxo Group Ltd Glaxo Wellcome House, Berkeley Avenue, Greenford, Middlesex UB6 0NN, Great Britain
Patents ADP number *(if you know it)* 473587003	
If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom
4. Title of the invention	DNA DOSAGE FORMS
5. Name of your agent *(if you have one)*	Corporate Intellectual Property
"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	GlaxoSmithKline Two New Horizons Court BRENTFORD Middlesex TW8 9EP
Patents ADP number *(if you know it)* 8088437001	
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7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application — Date of filing *(day / month / year)*
8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer yes if:* *a) any applicant named in part 3 is not an inventor, or* *b) there is an inventor who is named as an applicant, or* *c) any named applicant is a corporate body* *See note (d)*	

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Continuation sheets of this form	
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Claim(s)	1
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Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
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11. We request ~~the grant~~ of a patent on the basis of this application

Signature Date 25-Jan-02

R J Easeman

12. Name and daytime telephone number of person to contact in the United Kingdom — R J Easeman 020 80474407

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DNA dosage forms

The present invention relates to DNA formulations suitable for ballistic delivery into the skin of the human body. In particular the present invention provides DNA formulations suitable for ballistic administration of DNA pharmaceutical agents into the skin. The present invention provides a novel DNA pharmaceutical agent dosage form, having a dense core element which is coated with an amorphous solid reservoir medium containing the DNA pharmaceutical agent. The dense core element is preferably a small metal bead suitable for ballistic delivery of the agent into a cell, commonly such beads are roughly spherical gold or tungsten microbeads of an average particle size in the range of between 0.5 to 10 micrometers in diameter. Preferably the solid pharmaceutical reservoir medium coating the beads is an amorphous polyol, and preferably a carbohydrate such as trehalose or sucrose. The solid pharmaceutical reservoir medium may further comprise a stabilising agent that inhibits the degradative effects of free radicals, such as a free radical scavenger or a metal ion chelator. The most preferred DNA pharmaceutical agent dosage form of the present invention comprise a DNA plasmid, a free radical scavenger, and a metal ion chelator, in solid solution within an amorphous glassy carbohydrate reservoir medium which is coated onto a gold microbead. The ballistic delivery dosage forms of the present invention are storage stable, in that the DNA is stabilised in its supercoiled form, and only substantially release the DNA after administration to the skin. Furthermore, vaccine delivery devices for the administration of the DNAvaccines into the skin are provided, methods of their manufacture, and their use in medicine.

The skin represents a significant barrier to external agents. A summary of human skin is provided in Dorland's Illustrated Medical Dictionary, 28th Edition. Starting from the external layers, working inwards, the skin comprises the epithelium consisting of the stratum corneum and the viable epithelium, and underlying the epithelium is the dermis. The viable epithelium consists of four layers: Stratum corneum, Stratum lucidium, Stratum granulosum, Stratum spinosum, and Stratum basale. The *epithelium* (including all five layers) is the outermost non-vascular layer of the skin, and varies between 0.07 and 0.12 mm thick (70-120 μm). The epithelium is populated with keratinocytes, a cell that produces keratin and constitutes 95% of the dedicated epidermal cells. The other 5% of cells are melanocytes. The underlying

dermis is normally found within a range of 0.3 to about 3 mm beneath the surface of the stratum corneum, and contains sweat glands, hair follicles, nerve endings and blood vessels.

The stratum corneum dominates the skin permeability barrier and consists of a few dozen horny, keratinised epithelium layers. The narrow interstices between the dead or dying keratinocytes in this region are filled with crystalline lipid multilamellae. These efficiently seal the interstices between the skin or body interior and the surroundings by providing a hydrophobic barrier to entry by hydrophylic molecules. The stratum corneum being in the range of 30-70 μm thick.

Langerhans cells are found throughout the basal granular layer of the viable epithelium (stratum spinosum and stratum granulosum, (Small Animal Dermatology - Third Edition, Muller - Kirk – Scott, Ed: Saunders (1983)) and are considered to play an important role in the immune system's initial defence against invading organisms. This layer of the skin therefore represents a suitable target zone for certain types of vaccine.

Conventional modes for administration of pharmaceutical agents into or across the skin, most commonly by hypodermic needle and syringe, are associated with numerous disadvantages. Such disadvantages include pain, the requirement for trained professionals to administer the agent, and also the risk of needle-stick injuries to the administrator with the accompanying risk of infection with a blood born disease. As such, there is a need to improve the method of administration of all types of pharmaceutical into or through the skin.

A number of alternative approaches have been described in order to overcome the problems of administering agent across the stratum corneum, including various devices for the ballistic delivery of vaccines in supersonic gas flow.

DNA vaccines usually consist of a bacterial plasmid vector into which is inserted a strong viral promoter, the gene of interest which encodes for an antigenic peptide and a polyadenylation/transcriptional termination sequences. The gene of interest may encode a full protein or simply an antigenic peptide sequence relating to the pathogen, tumour or other agent which is intended to be protected against. The plasmid can be grown in bacteria, such as for example *E.coli* and then isolated and prepared in an appropriate medium, depending upon the intended route of administration, before being administered to the host. Following administration the

host cells produce the plasmid encoded protein or peptide. The plasmid vectors are generally made without an origin of replication which is functional in eukaryotic cells, in order to prevent plasmid replication in the mammalian host and integration within chromosomal DNA of the animal concerned. Information in relation to DNA vaccination is provided in Donnelly *et al* "DNA vaccines" *Ann. Rev Immunol.* 1997 15: 617-648, the disclosure of which is included herein in its entirety by way of reference.

Plasmid based delivery of genes, particularly for immunisation or gene therapy purposes is known. For example, administration of naked DNA by injection into mouse muscle is outlined in WO90/11092. Johnston et al WO 91/07487 describe methods of transferring a gene to veterbrate cells, by the use of microbeads onto which a polynucleotide encoding a gene of interest has been precipitated, and accelerating the DNA/microbeads such that they penetrate the target cell. Devices for administration of gold or tungsten beads coated with DNA into cells of the skin are described in US 5,630,796; WO 96/04947; WO 96/12513; WO 96/20022; WO 97/34652; WO 97/48485; WO 99/01168; WO 99/01169. Methods of vaccination using crystalline forms of ballistically delivered pharmaceutical agent are described in WO 99/27961. The present invention provides improved DNA dosage forms for use needleless ballistic delivery devices such as those described in the above publications. The formulations wherein DNA is precipitated onto gold beads as described in the art have the problem that it is difficult to co-formulate the DNA with additional agents/excipients. The present invention provides a method of co-formulation of additional agents.

Solid dosage forms comprising a pharmaceutical agent (including DNA plasmids) and a stabilising polyol (such as a sugar) wherein the dosage forms are in the form of ballistically delivered powders are described in WO 96/03978. The stabilisation of agents in amorphous sugar glasses has been described in US 5,098,893.

Sugars used in pharmaceutical formulations can be either crystalline or amorphous. Amorphous solids are distinguished from crytalline by their lack of three-dimensional long-range order found in crystalline materials. Amorphous solids are similar to liquids at a molecular level wherein the molecules are randomly arranged. Amorphous sugars impart stability to pharmaceutical formulations when stored at

temperatures below the glass transition temperature. Amorphous sugars exhibit a property in which there is a change in the mobility of the molecules in the sugar matrix below a temperature called the glass transition temperature. Below this temperature (Tg), amorphous sugars exist in a glassy state and above this temperature in a rubbery state. At temperatures below Tg the mobility of the sugar molecules and any molecules associated or trapped in the sugar matrix is extremely low giving rise to long-term stability of such formulations. In other words, the formation of a glass dramatically reduces the diffusional rates of the molecules. This is also accompanied by a decrease in the heat capacity at constant pressure (Cp) by 40 to 100%. This transition can be readily observed by sensitive thermal techniques like differential scanning calorimetry (Duddu, S.P., Zhang G, and Dal Monte, P. R., 1997., Pharm Res., 14: 596-600). The stabilizing properties of sugars have also been attributed to their hydrogen bonding properties with biological molecules like proteins.

It is desirable for DNA pharmaceutical agents to be delivered in a supercoiled form. Supercoiled DNA in liquid pharmaceutical preparations are known to degrade over time resulting in the loss of the supercoiled structure and associated formation of open circle or linear DNA structures (Evans *et al.*, 2000, Journal of Pharmaceutical Sciences, 89(1), 76-87; WO 97/40839). One mechanism by which this chain scission reaction may occur is oxidation of the DNA by free hydroxyl radicals produced from dissolved oxygen in the DNA solutions, a process that is catalysed by metal ions. The free radical formation reaction may be catalysed by several transition metal ions, the most common of which, however, are iron and copper ions (Fe^{+3}, Fe^{+2}, Cu^{+2} or Cu^{+1}; Evans *et al. supra*).

The instability of supercoiled DNA is apparent when the DNA is in liquid solution. However, removal of trace metal ions from supercoiled DNA containing liquid solutions with metal ion chelators, and/or mopping up free radicals in solution by non-reducing free radical scavengers stabilises the DNA in the supercoiled form and protects the DNA from oxidation (WO 97/40839). The problem of stabilisation of dry forms of DNA once coated onto a gold or tungsten bead has hitherto not been addressed in the art. Surprisingly, the present inventors have observed that dry forms of DNA when coated onto gold or tungsten microbeads are also unstable.

The present invention overcomes these problems and provides a DNA ballistic delivery dose which is capable of administering and releasing the DNA

agents efficiently into the skin, with or without additional excipients, and also in which the DNA is stabilised in its supercoiled form.

The present invention provides a novel DNA pharmaceutical agent dosage form, having a dense core element which is coated with an amorphous solid reservoir medium containing the DNA pharmaceutical agent.

Preferably the amorphous solid reservoir medium further comprises a stabilising agent that inhibits the degradative effects of free radicals. Preferably the stabilising agent is a free radical scavenger or a metal ion chelator.

The solid pharmaceutical reservoir medium is preferably an amorphous polyol, and preferably a carbohydrate such as trehalose or sucrose.

Most preferably the solid vaccines of the present invention comprise a DNA plasmid, a free radical scavenger, and a metal ion chelator, in solid solution within an amorphous glassy reservoir medium which is coated onto dense core element such as a gold microbead.

DNA vaccine delivery devices form a preferred aspect of the present invention. In such applications the agent to be delivered is a polynucleotide that encodes an antigen or antigens derivable from a pathogen such as micro-organisms or viruses, or may be a self antigen in the case of a cancer vaccine or other self antigen. The polynucleotide may be delivered alone or it may also comprise an agent to enhance uptake of the DNA into the cell, an adjuvant or other immunostimulant to improve and/or direct the immune response, and may also further comprise pharmaceutically acceptable excipient(s).

Certain embodiments of the device described herein also have the significant advantage of being stored at room temperature thus reducing logistic costs and releasing valuable refrigerator space for other products.

The solid amorphous reservoir medium is preferably a polyol that fulfils the function required for the present invention. The reservoir must be capable of adhering to the microbead to a sufficient extent that the reservoir remains physically stable and attached during prolonged storage, and also remains substantially intact during the administration procedure when the coated microbead is projected through the *stratum corneum*. The reservoir must also be capable of holding or containing a suspension or solution of agent to be delivered in any dry or partially dry form, which is released into the skin during biodegradation of the reservoir medium.

Biodegradation of the medium in the sense of the present invention means that the reservoir medium changes state, such that changes from its non-releasing to its releasing states whereby the agent enters into the skin. The release of the active agent may involve one or more physical and/or chemical processes such as hydration, diffusion, phase transition, crystallisation, dissolution, enzymatic reaction and/or chemical reaction. Depending on the choice of reservoir medium, biodegradation can be induced by one or more of the following: water, body fluids, humidity, body temperature, enzymes, catalysts and/or reactants. The change of the reservoir medium may therefore be induced by hydration, and warming associated with the higher humidity and temperature of the skin. The reservoir medium may then degrade by dissolution and/or swelling and/or change phase (crystalline or amorphous), thereby disintegrating or merely increase the permeation of the medium.

Preferably the medium dissolves, and is metabolised or expelled or excreted from the body, but the reservoir may alternatively remain attached to microbead which may be expelled from the body by several mechanisms including sloughing off of dead skin cells during normal skin replacement. Release of the agent by dissolution of the reservoir medium is preferred.

Suitable polyol reservoir media are as sugars, polysaccharides, substituted polyols such as hydrophobically derivatised carbohydrates, amino acids, biodegradable polymers or co-polymers such as poly(hydroxy acid)s, polyahhydrides, poly(ortho)esters, polyurethanes, poly(butyric acid)s, poly(valeric acid)s, and poly(lactide-co-caprolactone)s, or polylactide co-glycolide. The coating of the microblades is in the amorphous state, however the reservoir medium may also be partially amorphous and partially crystalline.

Particularly preferred reservoir media are those that stabilise the agent to be delivered over the period of storage. For example, antigen or agent dissolved or dispersed in a polyol glass or simply dried in a polyol are storage stable over prolonged periods of time (US 5,098,893, US 6,071,428; WO 98/16205; WO 96/05809; WO 96/03978; US 4,891,319; US 5,621,094; WO 96/33744). Such polyols form the preferred set of reservoir media.

Preferred polyols include sugars, including mono, di, tri, or oligo saccharides and their corresponding sugar alcohols. Suitable sugars for use in the present invention are well known in the art and include, trehalose, sucrose, lactose, fructose,

galactose, mannose, maltulose, iso-maltulose and lactulose, maltose, or dextrose and sugar alcohols of the aforementioned such as mannitol, lactitol and maltitol. Sucrose, Lactose, Raffinose and Trehalose are preferred.

The reservoir mediums of the present invention may preferably further contain a stabilising agent that inhibits the degradative effects of free radicals. Preferred stabilising agents include stabilising metal ion chelating agents, such preferred metal ion chelating agents include inositol hexaphosphate, tripolyphosphate, succinic and malic acid, ethylenediamine tetraacetic acid (EDTA), tromethamine (TRIS), Desferal, diethylenetriaminepentaacetic acid (DTPA) and ethylenediamindihydroxyphenylacetic acid (EDDHA). Other preferred stabilising agents are non-reducing free radical scavengers, and preferably such as agents are ethanol, methionine or glutathione. Other suitable chelators and scavengers (and those which are not suitable) may be readily identified by the man skilled in the art by routine experimentation (as described in WO 97/40839).

The preferred solid reservoir media in the devices of the present invention contain a metal ion chelating agent or a non-reducing free radical scavenger. Most preferably the solid reservoir media in the devices of the present invention contain both a metal ion chelating agent and a non-reducing free radical scavenger.

The amounts of the stabilising agents may be determined by the man skilled in the art, but generally are in the range of 0.1-10mM for the metal ion chelators, Ethanol is present in an amount up to about 5% (v/v), methionine is present at about 0.1 to 100mM and Glutathione is present at about 0.1 to 10% (v/v).

In addition to these stabilising agents, further steps may be taken to enhance the stability of the DNA in the solid vaccines. For example, the formulations may be made using solutions which themselves were demetalated before use (for example by using commercially available demetalating resin such as Chelex 100 from Biorad) and/or the formulation may be finalised in a high pH (such as pH 8-10).

Particularly preferred formulations which may be combined with DNA and coated onto the dense core elements to form solid dosage forms of the present invention contain polyols (preferably sucrose or trehalose) dissolved in demetalated water or Phosphate or Tris based buffers and further comprising either:

A. 10mM methionine and 2.9% ethanol, or

B. 3.7% ethanol and 1mM EDTA, or

C. 100mM Tris, 1mM EDTA and 10mM methionine and 2.9% ethanol, or

D. 100mM Tris, 1mM EDTA and 10mM methionine, or

E. 100mM Tris, 1mM EDTA and 2.9% ethanol.

In the preferred methods of manufacture of the present invention the DNA is stored and handled in these stabilising agents prior to final formulation with the sugar.

Preferably the DNA and stabilising agent are in a solid solution within the amorphous, and preferably glassy reservoir medium.

It is preferred that the reservoir medium forms an amorphous glass upon drying. The glass reservoir may have any glass transition temperature, but preferably it has a glass transition temperature that both stabilises the pharmaceutical agent during storage and also facilitates rapid release of the agent after insertion of the reservoir into the skin. Accordingly, the glass transition temperature is greater than 30-40°C, but most preferably is around body temperature (such as, but not limited to 37-50°C).

One major advantage of the present invention is the fact that the DNA present is stabilised so that upon release, it is largely in its supercoiled form. Amongst other factors this stability is primarily a result of the encapsulation of the DNA in the amorphous or glassy reservoir medium, and may be further enhanced by the presence of agents to counter the effects of free radicals.

Plasmid DNA stability can be defined in a number of ways and can be a relative phenomenon determined by the conditions of storage such as pH, humidity and temperature. For storage at pH $\geq$ 8.0 in the presence of iron ions on the coated reservoir, preferably >50% of plasmid remains supercoiled, (ccc, covalently closed circular), upon storage for 1 to 3 months at 4^0C. More preferably, under the storage conditions described, >60% of plasmid remains ccc and more preferably, under these storage conditions, >90% of plasmid remains ccc for 1 to 3 months at 4^0C. For coating on to non – metal ion based needles or microneedles, the stability of plasmid DNA, under the conditions described above, would be preferably >90% ccc, after 3 months storage at 25^0C. More preferably, under these storage conditions, >90% of plasmid remains ccc for 1 year at 25^0C, and more preferably >90% of plasmid remains ccc for 2 years at 25^0C.

Studies to determine plasmid stability are well known to those skilled in the art and are described in (Evans *et al., Supra;* WO 97/40839). These include techniques to

measure and quantify the percentage of supercoiled, ccc, plasmid DNA either by agarose gel electrophoresis, anion exchange HPLC, (Ferreira, G. *et al.*, 1999, *Pharm. Pharmacol. Commun.*, 5, pp57-59), or capillary gel electrophoresis, (Schmidt *et al.*, 1999, *Anal. Biochem.*, 274, 235-240). In addition enhanced stability of DNA can be assayed by measuring the quantity of DNA present over time by fluorescence, following Topology by Agarose gel electrophoresis, and also measuring in vitro and in vivo potency of the DNA vaccine.

In the context of the present invention the solid reservoir medium coats the core elements in a manner that the resultant formulation is suitable for administration by ballistic delivery devices. Accordingly each core element may be fully or partially covered by the reservoir, or a plurality of elements may be trapped within a matrix of solid reservoir. In a related method of producing the dosage forms of the present invention, a large quantity of reservoir encompassing a large number of core elements may be ground into smaller particles which are suitable for administration by ballistic delivery devices.

Other suitable excipients which may be included in the formulation include buffers, amino acids, phase change inhibitors ('crystal poisoners') which may be added to prevent phase change of the coating during procesing or storage or inhibitors to prevent deleterious chemical reactions during processing or storage such Maillard reaction inhibitors like amino acids.

The solid dosage forms of the present invention are used in ballistic transfection of skin cells using devices that entrain the DNA coated particles in a gas flow. The particles pass through the stratum corneum and enter into a cell where the DNA is released and expressed by the host cell. Alternatively, the particle enters the extracellular space and releases the DNA therein. Accordingly, the core elements that are suitable for use in the present invention are those that are suitable for this purpose. The core elements impart upon the final dosage form sufficient strength and momentum to pierce the stratum corneum in any given ballistic delivery device. It is preferred that the core elements have sufficient density to impart sufficient momentum to the DNA coated particles, suitable dense cores have been found to be gold or tungsten microbeads. The size of the core elements is preferably that which imparts sufficient mass to give the required momentum to the DNA coated particles, whilst not being too large such that the skin cells suffer too much damage. Suitable core element particle sizes are those that when coated form particles of a mean diameter in

the range of 0.5 to 100 μm, preferably between 1 to 50 μm, more preferably between 1 to 10μm, and most preferably around 2 μm in diameter.

In general the core elements are roughly spherical, although non-regular forms may be used. Most preferably the core elements are gold or tungsten microbeads.

The present invention claims that an amorphous sugar when present with metal particles and DNA will impart long-term stability to the formulation. Other excipients like surfactants and buffers may be included in the formulation.

Examples of methods for the preparation of such amorphous sugar containing formulations include:

1. Freeze-drying

Mix the solution containing sugar, DNA, gold particles and fill into glass vials. These vials are partially stoppered and loaded into a lyophilizer. The shelf temperature is then reduced to –45C leading to the product in the vials being frozen. After allowing all the vials to freeze, the condensor is chilled to sub –60C temperature. Primary drying is then carried out by raising the shelf temperature to approximately –30C while applying a vacuum of approximately 100 mT. During primary drying the water from the ice crystals that are formed is sublimated. After the primary drying is complete, the shelf temperature is raised to above ambient temperature and maximum vacuum is applied. The secondary drying removes any tightly bound water and dries the powder to achieve long term stability.

2. Spray-drying

Spray drying is a dehydration process that utilizes heat from a hot gas stream (usually air) to evaporate dispersed droplets created by atomization of a continuous liquid feed. Resulting powder products dry within a few seconds into fine particles. The feasibility of spray drying for generating therapeutic protein powders has been amply demonstrated ((Broadhead, J., Rouan, S.K.E., Hau, I., and Rhodes, C.T. 1994. J. Pharm. Pharmacol. 46: 458-467.; Mumenthaler, M., Hsu, C.C., and Pearlman, R. 1994. Pharm. Res. 11: 12-20)). In such an application to our formulation mixtures, the formulated DNA, gold particles and sugar solution will be fed into a spray dryer with a typical inlet temperature in the range of 50 to 150C typically at a flow rate

between 0.1 and 10 mL/min. The resulting powder is dry and is collected from the collection chamber.

3. Spray freeze-drying

Spray freeze-drying is a process in which the solution containing the DNA, gold particles and sugars is sprayed onto trays containing dry ice or liquid nitrogen. This results in the instantaneous freezing of the droplets. The trays are then loaded into a lyophilizer and the particles are then freeze-dried according to the process described above.

Using these techniques each solid DNA delivery may be loaded with relatively high amounts of DNA.

Preferably the vaccine formulations of the present invention contain DNA that encodes an antigen or antigenic composition capable of eliciting an immune response against a human pathogen, which antigen or antigenic composition is derived from HIV-1, (such as tat, nef, gp120 or gp160), human herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2, cytomegalovirus ((esp Human)(such as gB or derivatives thereof), Rotavirus (including live-attenuated viruses), Epstein Barr virus (such as gp350 or derivatives thereof), Varicella Zoster Virus (such as gpI, II and IE63), or from a hepatitis virus such as hepatitis B virus (for example Hepatitis B Surface antigen or a derivative thereof), hepatitis A virus, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as paramyxoviruses: Respiratory Syncytial virus (such as F and G proteins or derivatives thereof), parainfluenza virus, measles virus, mumps virus, human papilloma viruses (for example HPV6, 11, 16, 18, ..), flaviviruses (e.g. Yellow Fever Virus, Dengue Virus, Tick-borne encephalitis virus, Japanese Encephalitis Virus) or Influenza virus (whole live or inactivated virus, split influenza virus, grown in eggs or MDCK cells, or Vero cells or whole flu virosomes (as described by R. Gluck, Vaccine, 1992, 10, 915-920) or purified or recombinant proteins thereof, such as HA, NP, NA, or M proteins, or combinations thereof), or derived from bacterial pathogens such as *Neisseria spp*, including *N. gonorrhea* and *N. meningitidis* (for example capsular polysaccharides and conjugates thereof, transferrin-binding proteins, lactoferrin binding proteins, PilC, adhesins); *S. pyogenes* (for example M proteins or fragments thereof, C5A protease, lipoteichoic acids), *S. agalactiae, S. mutans; H.*

ducreyi; Moraxella spp, including M catarrhalis, also known as Branhamella catarrhalis (for example high and low molecular weight adhesins and invasins*); Bordetella spp, including B. pertussis* (for example pertactin, pertussis toxin or derivatives thereof, filamenteous hemagglutinin, adenylate cyclase, fimbriae), *B. parapertussis and B. bronchiseptica; Mycobacterium spp., including M. tuberculosis* (for example ESAT6, Antigen 85A, -B or -C), *M. bovis, M. leprae, M. avium, M. paratuberculosis, M. smegmatis; Legionella spp,* including *L. pneumophila; Escherichia spp,* including *enterotoxic E. coli* (for example colonization factors, heat-labile toxin or derivatives thereof, heat-stable toxin or derivatives thereof), enterohemorragic *E. coli,* enteropathogenic *E. coli* (for example shiga toxin-like toxin or derivatives thereof); *Vibrio spp,* including *V. cholera* (for example cholera toxin or derivatives thereof*); Shigella spp,* including *S. sonnei, S. dysenteriae, S. flexnerii; Yersinia spp,* including *Y.* enterocolitica (for example a Yop protein) , *Y. pestis, Y. pseudotuberculosis; Campylobacter spp,* including *C. jejuni* (for example toxins, adhesins and invasins) and *C. coli; Salmonella spp,* including *S. typhi, S. paratyphi, S. choleraesuis, S. enteritidis; Listeria spp.,* including *L. monocytogenes; Helicobacter spp,* including *H.* pylori (for example urease, catalase, vacuolating toxin); *Pseudomonas spp,* including *P. aeruginosa; Staphylococcus spp.,* including *S. aureus, S. epidermidis; Enterococcus spp.,* including *E. faecalis, E. faecium; Clostridium spp.,* including *C.* tetani (for example tetanus toxin and derivative thereof), *C. botulinum* (for example botulinum toxin and derivative thereof*), C. difficile* (for example clostridium toxins A or B and derivatives thereof); *Bacillus spp.,* including *B. anthracis* (for example botulinum toxin and derivatives thereof*); Corynebacterium spp.,* including *C. diphtheriae* (for example diphtheria toxin and derivatives thereof); *Borrelia spp.,* including *B. burgdorferi* (for example OspA, OspC, DbpA, DbpB), *B. garinii* (for example OspA, OspC, DbpA, DbpB), *B. afzelii* (for example OspA, OspC, DbpA, DbpB*), B. andersonii* (for example OspA, OspC, DbpA, DbpB), *B. hermsii; Ehrlichia spp.,* including *E. equi* and the agent of the Human Granulocytic Ehrlichiosis*; Rickettsia spp,* including *R. rickettsii; Chlamydia spp., including C. trachomatis* (for example MOMP, heparin-binding proteins), *C. pneumoniae* (for example MOMP, heparin-binding proteins*), C. psittaci; Leptospira spp.,* including *L. interrogans; Treponema spp.,* including *T. pallidum* (for example the rare outer membrane proteins*), T. denticola, T. hyodysenteriae;* or derived from parasites such as

Plasmodium spp., including *P. falciparum; Toxoplasma spp.*, including *T. gondii (for example SAG2, SAG3, Tg34); Entamoeba spp.*, including *E. histolytica; Babesia spp.*, including *B. microti; Trypanosoma spp.*, including *T. cruzi; Giardia spp.*, including *G. lamblia; Leshmania spp.*, including *L. major; Pneumocystis spp.*, including *P. carinii; Trichomonas spp.*, including *T. vaginalis; Schisostoma spp.*, including *S. mansoni,* or derived from yeast such as *Candida spp.*, including *C. albicans; Cryptococcus spp.*, including *C. neoformans.* Other preferred bacterial vaccines comprise antigens derived from *Haemophilus spp., including H. influenzae type B* (for example PRP and conjugates thereof), *non typeable H. influenzae,* for example OMP26, high molecular weight adhesins, P5, P6, protein D and lipoprotein D, and fimbrin and fimbrin derived peptides (US 5,843,464).

In another embodiment of the present invention the DNA dosage form contains a DNA vaccine in combination with a non-DNA antigen such as a protein or polysaccharide antigen derived from a pathogen.

Vaccines of the present invention, may advantageously also include an immunologically effective adjuvant in solid solution together with the DNA. Alternatively the adjuvant may be associated with separate microbeads to the DNA coated microbead. Suitable adjuvants for vaccines of the present invention comprise those adjuvants that are capable of enhancing the antibody responses against the immunogen. Suitable immunostimulatory agents include, but this list is by no means exhaustive and does not preclude other agents: synthetic imidazoquinolines such as imiquimod [S-26308, R-837], (Dockrell and Kinghorn, 2001, Journal of Antimicrobial Chemotherapy, 48, 751-755; Harrison, et al. 'Reduction of recurrent HSV disease using imiquimod alone or combined with a glycoprotein vaccine', Vaccine 19: 1820-1826, (2001)); and resiquimod [S-28463, R-848] (Vasilakos, et al. ' Adjuvant activites of immune response modifier R-848: Comparison with CpG ODN', Cellular immunology 204: 64-74 (2000).), Schiff bases of carbonyls and amines that are constitutively expressed on antigen presenting cell and T-cell surfaces, such as tucaresol (Rhodes, J. et al. ' Therapeutic potentiation of the immune system by costimulatory Schiff-base-forming drugs', Nature 377: 71-75 (1995)), cytokine, chemokine and co-stimulatory molecules as either protein or peptide, this would include pro-inflammatory cytokines such as GM-CSF, IL-1 alpha, IL-1 beta, TGF-alpha and TGF – beta, Th1 inducers such as interferon gamma, IL-2, IL-12, IL-15 and

IL-18, Th2 inducers such as IL-4, IL-5, IL-6, IL-10 and IL-13 and other chemokine and co-stimulatory genes such as MCP-1, MIP-1 alpha, MIP-1 beta, RANTES, TCA-3, CD80, CD86 and CD40L, , other immunostimulatory targeting ligands such as CTLA-4 and L-selectin, apoptosis stimulating proteins and peptides such as Fas, (49), synthetic lipid based adjuvants, such as vaxfectin, (Reyes et al., 'Vaxfectin enhances antigen specific antibody titres and maintains Th1 type immune responses to plasmid DNA immunization', Vaccine 19: 3778-3786) squalene, alpha- tocopherol, polysorbate 80, DOPC and cholesterol, endotoxin, [LPS], Beutler, B., 'Endotoxin, 'Toll-like receptor 4, and the afferent limb of innate immunity', Current Opinion in Microbiology 3: 23-30 (2000)) ; CpG oligo- and di-nucleotides, Sato, Y. et al., 'Immunostimulatory DNA sequences necessary for effective intradermal gene immunization', Science 273 (5273): 352-354 (1996). Hemmi, H. et al., 'A Toll-like receptor recognizes bacterial DNA', Nature 408: 740-745, (2000) and other potential ligands that trigger Toll-like receptors to produce Th1-inducing cytokines, such as synthetic Mycobacterial lipoproteins, Mycobacterial protein p19, peptidoglycan, teichoic acid and lipid A.

Certain preferred adjuvants for eliciting a predominantly Th1-type response include, for example, a Lipid A derivative such as monophosphoryl lipid A, or preferably 3-de-O-acylated monophosphoryl lipid A. MPL® adjuvants are available from Corixa Corporation (Seattle, WA; *see*, for example, US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555, WO 99/33488 and U.S. Patent Nos. 6,008,200 and 5,856,462. Immunostimulatory DNA sequences are also described, for example, by Sato et al., *Science 273*:352, 1996. Another preferred adjuvant comprises a saponin, such as Quil A, or derivatives thereof, including QS21 and QS7 (Aquila Biopharmaceuticals Inc., Framingham, MA); Escin; Digitonin; or *Gypsophila* or *Chenopodium quinoa* saponins.

In this aspect of the present invention the preferred immunostimulatory agent or adjuvant is immiquimod or other related molecules (such as resiquimod) as described in PCT patent application publication number WO 94/17043 (the contents of which are incorporated herein by reference).

The amount of expressible DNA in each vaccine administration is selected as an amount which induces an immunoprotective response without significant adverse side effects in typical vaccinees. Such amount will vary depending upon which specific DNA construct is employed, however, it is expected that each dose will generally comprise 1-1000 μg of DNA, preferably 1-500 μg, more preferably 1-100 μg, of which 1 to 50μg is the most preferable range. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

Also provided by the present invention are ballistic delivery devices loaded with the DNA dosage forms of the present invention.

The formulations of the present invention may be used for both prophylactic and therapeutic purposes. Accordingly, the present invention provides for a method of treating a mammal susceptible to or suffering from an infectious disease or cancer, or allergy, or autoimmune disease. In a further aspect of the present invention there is provided a vaccine as herein described for use in medicine. Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978.

The present invention is exemplified by, but not limited to, the following examples.

Example 1, *Demonstration of coating of microbeads with a reservoir medium comprising plasmid DNA.*

Plasmid preparation and formulations.

The plasmids used in this study are all shown in Figure 1.

pEGFP-C1 is a GFP expression vector, (Clontech, Palo Alto, California, USA).

pGL3CMV is a luciferase expression vector based upon pGL3 Basic, (Promega Corporation., Madison, Wisconsin, USA), where the CMV immediate early promoter drives luciferase expression.

pVAC1.ova is a chicken ovalbumin expression plasmid, constructed by ligating PCR amplified cDNA encoding chicken ovalbumin from pUGOVA, into the expression

vector pVAC1. pVAC1 is a modification of the mammalian expression vector, pCI, (Promega), where the multiple cloning site, from EcoRI to Bst ZI, has been replaced by the EMCV IRES sequence flanked 5' by unique Nhe I, Rsr II and Xho I and 3' by unique Pac I, Asc I and Not I restriction enzyme sites, amplified from pGL3Basic, (Promega). Supercoiled plasmid DNA, (low endotoxin), was purified on a large scale, aproximately 100mg yield, to high purity using a combination of alkaline SDS lysis, ultrafiltration and anion exchange column chromatography.

Plasmids were resuspended in TE, (10mM TrisHCl, 1mM EDTA), pH 8.0 at 1ug / ul. And determined as >95% supercoiled upon analysis by agarose gel electrophoresis.

Plasmids were formulated in a variety of solutions, for coating needles, by a standard large-scale ethanol precipitation procedure. The precipitated DNA was resuspended directly into the aqueous formulation solutions at concentrations of 0.5 to 12 ug/ul, (See Chapter 1, Molecular Cloning: A Laboratory Manual, Sambrook, J. *et al.*, 2nd Edition, 1989, CSH laboratory Press, Cold Spring Harbor, New York, USA).

1.2 Freeze-drying

Mix the solution containing sugar (between 1-40% sucrose or trehalose), DNA plasmid, gold particles and fill into glass vials. These vials are partially stoppered and loaded into a lyophilizer. The shelf temperature is then reduced to –45C leading to the product in the vials being frozen. After allowing all the vials to freeze, the condensor is chilled to sub –60C temperature. Primary drying is then carried out by raising the shelf temperature to approximately –30C while applying a vacuum of approximately 100 mT. During primary drying the water from the ice crystals that are formed is sublimated. After the primary drying is complete, the shelf temperature is raised to above ambient temperature and maximum vacuum is applied. The secondary drying removes any tightly bound water and dries the powder to achieve long term stability.

1.3 Spray-drying

Spray drying is a dehydration process that utilizes heat from a hot gas stream (usually air) to evaporate dispersed droplets created by atomization of a continuous liquid feed. Resulting powder products dry within a few seconds into fine particles. The feasibility of spray drying for generating therapeutic protein powders has been amply

demonstrated ((Broadhead, J., Rouan, S.K.E., Hau, I., and Rhodes, C.T. 1994. J. Pharm. Pharmacol. 46: 458-467.; Mumenthaler, M., Hsu, C.C., and Pearlman, R. 1994. Pharm. Res. 11: 12-20)). In such an application to our formulation mixtures, the formulated DNA, gold particles and sugar solution will be fed into a spray dryer with a typical inlet temperature in the range of 50 to 150C typically at a flow rate between 0.1 and 10 mL/min. The resulting powder is dry and is collected from the collection chamber.

1.4. Spray freeze-drying

Spray freeze-drying is a process in which the solution containing the DNA, gold particles and sugars is sprayed onto trays containing dry ice or liquid nitrogen. This results in the instantaneous freezing of the droplets. The trays are then loaded into a lyophilizer and the particles are then freeze-dried according to the process described above.

The formulations resulting from the above techniques may be used directly or after milling in conventional ballistic delivery devices, and expression in target cells may be followed by observing luciferase expression. The samples are also stable as measured by maintenance of supercoiled structure.

Claims

1. A DNA pharmaceutical agent dosage form, having a dense core element coated with an amorphous solid reservoir medium containing the DNA pharmaceutical agent.

2. A DNA pharmaceutical agent dosage form as claimed in claim 1 further comprising a stabilising agent that inhibits the degradative effects of free radicals.

3. A DNA pharmaceutical agent dosage form as claimed in claim 2 wherein the stabilising agent is one or both of a metal ion chelator and a free radical scavenger.

4. A DNA pharmaceutical agent dosage form as claimed in claims 1, 2 or 3 wherein the solid biodegradable reservoir medium is a polyol.

5. A DNA pharmaceutical agent dosage form as claimed in claim 4, wherein the polyol is a stabilising polyol.

6. A DNA pharmaceutical agent dosage form as claimed in any one of claims 1 to 5 wherein the solid biodegradable reservoir medium is a sugar.

7. A DNA pharmaceutical agent dosage form as claimed in claim 6 wherein the sugar is selected from lactose, sucrose, raffinose or trehalose.

8. A DNA pharmaceutical agent dosage form as claimed in any one of claims 1 to 6 wherein the solid biodegradable reservoir medium is in the form of a glass.

9. A DNA pharmaceutical agent dosage form as claimed in any one of claims 1 to 8, wherein the DNA pharmaceutical agent is a vaccine.

10. A DNA pharmaceutical agent delivery device as claimed in any one of claims 1 to 9, wherein the DNA is stabilised in a supercoiled form.

11. A DNA pharmaceutical agent dosage form, as claimed in claim 1 wherein the dense core elements are microbeads of a mean particle diameter of between 0.5 to 10 μm.

12. A DNA pharmaceutical agent dosage form as claimed in claim 11, wherein the dense core element is a gold or tungsten microbead.

Abstract

The present invention relates to DNA formulations suitable for ballistic delivery into the skin of the human body. In particular the present invention provides DNA formulations suitable for ballistic administration of DNA vaccines into the skin. The present invention provides a novel DNA pharmaceutical agent dosage form, having a dense core element which is coated with an amorphous solid reservoir medium containing the DNA pharmaceutical agent.